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Secretion of Flavins by *Shewanella* Species and Their Role in Extracellular Electron Transfer[▽]

Harald von Canstein,[1]* Jun Ogawa,[2] Sakayu Shimizu,[2] and Jonathan R. Lloyd[1]

School of Earth, Atmospheric and Environmental Sciences, The University of Manchester, Manchester, United Kingdom,[1] *and Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto, Japan*[2]

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Fe(III)-respiring bacteria such as *Shewanella* species play an important role in the global cycle of iron, manganese, and trace metals and are useful for many biotechnological applications, including microbial fuel cells and the bioremediation of waters and sediments contaminated with organics, metals, and radionuclides. Several alternative electron transfer pathways have been postulated for the reduction of insoluble extracellular subsurface minerals, such as Fe(III) oxides, by *Shewanella* species. One such potential mechanism involves the secretion of an electron shuttle. Here we identify for the first time flavin mononucleotide (FMN) and riboflavin as the extracellular electron shuttles produced by a range of *Shewanella* species. FMN secretion was strongly correlated with growth and exceeded riboflavin secretion, which was not exclusively growth associated but was maximal in the stationary phase of batch cultures. Flavin adenine dinucleotide was the predominant intracellular flavin but was not released by live cells. The flavin yields were similar under both aerobic and anaerobic conditions, with total flavin concentrations of 2.9 and 2.1 μmol per gram of cellular protein, respectively, after 24 h and were similar under dissimilatory Fe(III)-reducing conditions and when fumarate was supplied as the sole electron acceptor. The flavins were shown to act as electron shuttles and to promote anoxic growth coupled to the accelerated reduction of poorly crystalline Fe(III) oxides. The implications of flavin secretion by *Shewanella* cells living at redox boundaries, where these mineral phases can be significant electron acceptors for growth, are discussed.

Shewanella species are facultative anaerobic bacteria with a unique respiratory versatility (30), as they are able to couple the oxidation of organic substrates or hydrogen to the reduction of a wide variety of electron acceptors. The greatest energy yield can be gained with oxygen as the terminal electron acceptor, but in its absence, in the anoxic zones of lakes or in sediments, *Shewanella* species can exploit a wide range of alternative electron acceptors, including Fe(III) and Mn(IV) (12, 16). However, these metals are highly insoluble at neutral pH in most environments, posing the unique problem of how to conserve energy for growth through the transfer of electrons to an extracellular mineral surface (14). At least two distinct pathways have been proposed for electron transfer to the mineral substrate, namely, the direct transfer of electrons from the cell surface and the use of low-molecular-weight soluble redox mediators or "electron shuttles" to promote extracellular electron transfer (reviewed in reference 12). Direct electron transfer at the mineral-microbe interface is thought to involve a network of *c*-type cytochromes recently identified in the genome of *Shewanella oneidensis* MR-1 (8) and shown in several studies to be localized in the periplasm (25), in the outer membranes of *Shewanella* species (1, 5, 14), and in pilus-like assemblages (7). Thus, electron transfer from these outer membrane cytochromes or from alternative surface-associated protein structures noted in other Fe(III)-reducing prokaryotes (24) to insoluble minerals is a major factor potentially limiting the growth of Fe(III)-reducing prokaryotes that are not in direct contact with the mineral assemblage, for example, in a biofilm, or are separated from Fe(III) and Mn(IV) in occluded pore spaces in sediments. The second mechanism, the use of highly soluble redox-active electron shuttles that transfer electrons from cell-associated reductases to the mineral surface, obviates the need for direct contact. Early reports showed that exogenous redox shuttles, such as quinone-containing humic acids, promote the dissimilatory reduction of Fe(III) oxides (11), while more recent studies (19, 33) have suggested that *Shewanella* cells may even have the capability to produce and secrete endogenous electron shuttles themselves to promote the reduction of Fe(III) minerals, although such compounds have yet to be identified. The aim of this study was to identify the electron shuttle(s) secreted by *Shewanella* species and to confirm its potential to mediate the dissimilatory reduction of insoluble Fe(III) oxides.

MATERIALS AND METHODS

Bacterial strains and media. *Shewanella* strains Hac318, Hac319, Hac326, Hac334, Hac353, and Hac411 were isolated from dye-works wastewater by growth on Luria-Bertani solid medium and decoloration of the amended azo dye Direct Blue 53 (100 μM; Wako, Japan) and were identified by 16S rRNA gene sequencing. The DNA was amplified by PCR using a T Gradient thermal cycler (Biometra, Gottingen, Germany). The PCR mixture comprised, in a total volume of 50 μl, 5 ng of genomic DNA as a template, 200 pmol of each primer (27F and 1544R), 200 nmol of deoxynucleoside triphosphates, and 0.5 unit of Ex *Taq* polymerase (Takara-Bio, Otsu, Japan). One thermal cycle consisted of 94°C for 1 min, 55°C for 30 s, and 72°C for 1 min. A total of 30 cycles were performed. The PCR product was cloned with pT7Blue vector (Novagen). The nucleotides of the selected clones were sequenced by the dideoxy chain termination method, using a CEQ dye terminator cycle se-

* Corresponding author. Mailing address: The University of Manchester, SEAES, Oxford Road, Manchester M13 9PL, United Kingdom. Phone: 44-161-275 3800. Fax: 44-161-275 3947. E-mail: canstein@manchester.ac.uk.

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quencing kit with a CEQ2000XL automated sequencer DNA analysis system (Beckman Coulter). Sequence data were analyzed with Genetyx-Mac 11.2 (Software Development, Tokyo, Japan). The closest known relative to strains Hac318 (GenBank accession number DQ307730), Hac334 (GenBank accession number DQ307729), and Hac411 (GenBank accession number DQ307731) was *Shewanella putrefaciens* LMG 2369 (99% similarity over 1,454, 1,446, and 1,454 bases, respectively), while strains Hac319 (GenBank accession number DQ307732), Hac326 (GenBank accession number DQ307733), and Hac353 (GenBank accession number DQ307734) were most closely related to *Shewanella oneidensis* MR-1 (99% similarity over 1,454 bases each). *Shewanella* sp. strain J18 143 was isolated from soil contaminated with textile wastewater (17). The type strain *Shewanella oneidensis* MR-1 was isolated from Lake Oneida sediments (16). The *Shewanella baltica* strains Os155 and Os195 were isolated from Baltic Sea water (35), and *Shewanella frigidimarina* strain NCIMB400 was isolated from the North Sea (21). *S. frigidimarina* cultures were grown at a maximum temperature of 25°C. *Escherichia coli* JM109 (34), *Pseudomonas putida* Spi3 (32), *Pseudomonas stutzeri* Ibu8 (31), and *Pseudomonas aeruginosa* Bro12 (31) were used for comparison. The *Shewanella* mineral medium (SMM) used for aerobic and anaerobic growth was based on the medium used by Myers and Nealson (16), but without the addition of Casamino Acids. The electron donor used for anaerobic growth in SMM was DL-lactate (100 mM), unless otherwise indicated, while the electron acceptor was either fumarate, Fe(III)-citrate, or poorly crystalline Fe(III) oxide (26). The pH of all media was 7, and all chemicals were purchased from Sigma unless noted otherwise.

Isolation and identification of extracellular flavins. (i) Redox mediator assay. The presence of redox mediators was determined by testing for enhanced rates of decoloration of the azo dye Direct Blue 53. The assay mixtures were prepared aerobically, in triplicate, in 96-well microplates. For each assay, 40 μl of the sample was put into a well and decoloration was started by adding 40 μl of a freshly prepared mix containing potato dextrose broth (final concentration of potato starch, 4 g/liter; final concentration of dextrose, 20 g/liter) (Difco, Japan), piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) buffer (final concentration, 0.1 M) (Wako, Japan), Direct Blue 53 (final concentration, 0.1 mM; Wako, Japan), and *Shewanella* strain Hac334 cells (final optical density at 600 nm, approximately 0.2). The cells were harvested from mid-log phase in an aerobic potato dextrose broth culture, washed in phosphate-buffered saline (PBS), and kept on ice before being added to the assay mix. After the addition of the assay mix, the microplate was immediately transferred into an anaerobic cabinet (nitrogen atmosphere with 2% H_2), and the change in absorbance at 595 nm was measured with a microplate reader (Bio-Rad 550; Bio-Rad, Hercules, CA). No more than 5 min passed between mixing of the sample with the assay mix and the first photometer reading. The decoloration rate was calculated from the decrease in absorption at 595 nm over time. Since the cells reduced and decolored the dye without the addition of a redox mediator, samples were considered redox active when their decoloration rate was at least 10% above that of a mediator-free control.

(ii) Isolation of extracellular redox mediators. *Shewanella oneidensis* MR-1 and *Shewanella* strain Hac334 were grown aerobically or anaerobically for 24 h in SMM with 100 mM lactate and 20 mM fumarate. Biomass was removed by centrifugation (3 min, 10,000 × *g*); supernatants (500 μl each) were fractionated using reversed-phase high-performance liquid chromatography (HPLC) (Gemini 5u C18 110A 250- by 10.0-mm column [Phenomenex, United Kingdom] fitted to a GP50 gradient pump and a UVD170U UV-visible detector [both from Dionex, United Kingdom]). Separation was achieved with a nonlinear gradient of increasing methanol concentrations. Fractions (1.0 ml) were screened for the presence of a redox mediator with the redox mediator assay. The redox-active fractions were pooled, freeze-dried, and resuspended in double-distilled H_2O (ddH_2O), and constituent compounds were separated by reversed-phase HPLC employing a nonlinear gradient of methanol versus an aqueous solution of 20 mM ammonium acetate, pH 5.4. An initial isocratic step at 5% methanol for 6 min was followed by increases to 34.5% methanol (by time [*t*] = 12 min), 37% methanol (at 28 min), and 95% methanol (maintained at 33 to 38 min), followed by a rapid drop to 5% methanol (by *t* = 39 min), which was maintained for a further 16 min. The eluate was collected in fractions containing only one peak as monitored at 275 nm, and the fractions were screened for redox mediators as described previously. Redox mediator activity was detected predominantly in fractions containing the peaks eluted at 28 and 33 min.

(iii) Identification of extracellular redox mediators. The isolated and purified redox-active compounds were analyzed by UV-visible spectroscopy (Specord S600; Analytik Jena, Jena, Germany) and liquid chromatography-mass spectrometry (LC-MS) (LC-10A system with LC-MS 2010A column; Shimadzu, Kyoto, Japan). Internet searches (www.pubmed.gov; www.sigmaaldrich.com) were also used to find organic molecules with molecular sizes similar to those found by LC-MS, and matching molecules, including flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and riboflavin standards, were purchased (Sigma, United Kingdom) and compared to the purified compounds by using the techniques described above.

Isolation and identification of intracellular flavins. Cells were grown in SMM as described above for extracellular flavin analysis. Biomass from 100-ml cultures was harvested by centrifugation (20 min, 5,000 × *g*, 4°C), washed in 12 ml HEPES buffer (100 mM, pH 7.4; Sigma, United Kingdom), split into aliquots of 1.0 ml, pelleted again (3 min, 10,000 × *g*, 4°C), and kept on ice until the start of one of the following two cell lysis protocols.

(i) Alkaline lysis. The cells were resuspended in 490 μl NaOH (0.1 M), mixed thoroughly for 10 seconds, and then acidified by the addition of 510 μl HCl (0.1 M) to stabilize the flavins.

(ii) GTC lysis. The cells were resuspended in 100 μl GES buffer (5.0 M guanidium thiocyanate [GTC], 0.1 M EDTA, 0.5% Sarkosyl, pH 8.0) and mixed thoroughly for 10 seconds (23). The sample was normalized to a volume of 1.0 ml by adding 900 μl chilled ddH_2O.

Flavin analysis of cell lysates by HPLC followed the same procedure as that for the analysis of supernatants. Redox mediator activity was detected predominantly in fractions containing the peaks eluted at 25 and 28 min. The GTC and alkaline lysis protocols resulted in similarly high concentrations of flavins, with the alkaline lysis data being reported in this paper.

Quantification of flavins. FAD, FMN, and riboflavin (Sigma, United Kingdom) standard solutions of 0.01, 0.1, 1.0, 10.0, and 100 μM were analyzed separately using HPLC, and the peak area was calculated using Chromeleon software (version 6.50; Dionex). The flavin concentrations of unknown samples were calculated by comparison to a graph prepared for the standards.

Growth with poorly crystalline Fe(III) oxide and Fe(III) reduction assay. *Shewanella oneidensis* MR-1 and *Shewanella* strain Hac334 cells were grown anaerobically in nitrogen-flushed, oxygen-free SMM [pH 6.4; 100 mM lactate and 20 mM Fe(III)-citrate or fumarate] and washed twice with oxygen-free PBS to remove all traces of electron shuttles. The washed cells (500 μl) were inoculated into nitrogen-flushed, oxygen-free SMM (pH 6.4; 80 ml medium in 100-ml serum bottles) containing 100 mM lactate and 35 mM poorly crystalline Fe(III) oxide. Electron shuttles were added to the concentrations indicated and then incubated along with an electron shuttle-free control at 30°C. Experiments were performed in triplicate. Growth was determined as the increase in the number of viable cells, measured as CFU (see below).

Fe(II) was assayed spectrophotometrically after reaction with ferrozine based on the method of Lovley and Phillips (13). For the dissolution of mineral precipitates, 200 μl of sample was mixed with 800 μl of 1 M HCl and incubated for 1 hour at 20°C. Samples (10 μl) were mixed with 990 μl of ferrozine solution (2 mM in 50 mM HEPES buffer, pH 7) 60 seconds before measuring the absorption at 562 nm (Specord S600; Analytik Jena). Assays were performed in triplicate.

Reduction of poorly crystalline Fe(III) oxide with chemically reduced flavins. Flavins were reduced chemically by flushing a 10-ml solution containing 1.0 mM of FMN or riboflavin and 2.2 g of aluminum pellets coated with 0.5% palladium (BBL GasPak catalyst replacement charges; Becton Dickinson, MD) with hydrogen (1 ml/min) for 20 min. In control experiments, ddH_2O was subjected to the same treatment. The Al-Pd catalyst was removed by centrifugation. To 1,470 μl of the supernatants and 1,470 μl of untreated ddH_2O, 30 μl of poorly soluble Fe(III) was added, to a final Fe concentration of approximately 6 mM. The reaction mixtures were incubated for 24 h in an anaerobic cabinet in the dark at 20°C. Fe(II) was assayed as described above. For determination of the total Fe concentration, 100 μl of sample was pretreated with 200 μl of $H_3NO \cdot HCl$ (6.25 M) and 700 μl of HCl (0.5 M). Assays for Fe(II) by ferrozine were performed in triplicate.

Determination of cellular growth, viable cell numbers, and protein concentrations. Cellular growth was measured routinely as an increase in the optical density at 600 nm. Viable cell numbers were estimated by counting CFU. Samples were serially diluted in PBS (K_2HPO_4, 1.7 g/liter; KH_2PO_4, 1.3 g/liter; NaCl, 10 g/liter; pH 7). For each dilution step, 100 μl was spread onto tryptic soy broth plates solidified with 1.5% agar and incubated for 20 h at 30°C. All measurements were done in triplicate and were recorded only when between 10 and 400 colonies were counted per plate.

Protein concentrations in samples of whole-cell cultures were measured using a bicinchoninic acid-copper sulfate kit (Sigma, United Kingdom). Protein concentrations of unknown samples were calculated according to protein standard solutions ranging from 0.1 to 1.0 g/liter.

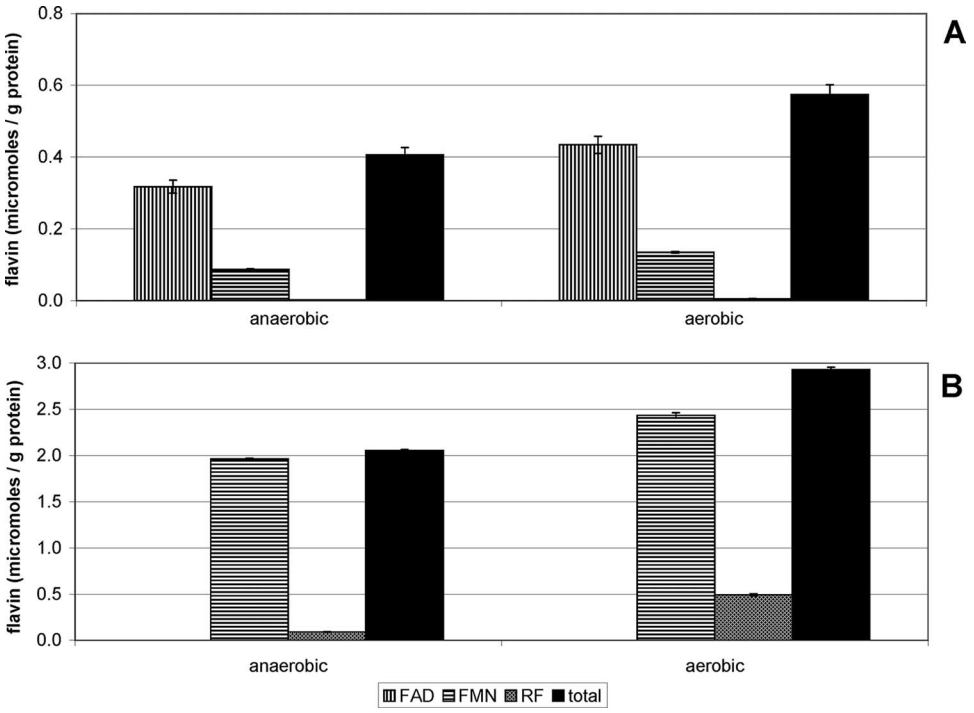

FIG. 1. Production of intracellular (A) and extracellular (B) flavins in *Shewanella oneidensis* MR-1 cultures grown anaerobically and aerobically for 24 h in minimal medium containing 50 mM lactate and 100 mM fumarate. Data are averages for triplicate cultures.

RESULTS

Purification and identification of extracellular electron shuttles. To identify potential soluble electron shuttling compounds secreted by *Shewanella* cultures, supernatants were collected from aerobic and anaerobic cultures of *Shewanella oneidensis* MR-1 and *Shewanella* strain Hac334 grown in minimal medium. The supernatants of *Shewanella* strain Hac334 cultures were fractionated using reversed-phase HPLC and screened for the presence of a redox mediator by testing for enhanced rates of decoloration of the azo dye Direct Blue 53 by the organism. In addition to promoting the reduction of insoluble metals, azo dye reduction by *Shewanella* species is also enhanced by the addition of electron shuttling compounds (22), making enhanced reduction and decoloration of azo dyes a suitable rapid screen for the presence of secreted redox mediators. The rate of decoloration by *Shewanella* cells was enhanced most by addition of the fraction eluted by 35% methanol. This was also the case for fractions from cultures of *S. oneidensis* MR-1.

The bright yellow fraction eluted by 35% methanol was purified with another HPLC step, employing a slow increase from 34.5 to 37% methanol, revealing two yellow, redox-active compounds, in the fractions at 28 min and at 33 min. Riboflavin and FMN standards behaved identically to the respective redox-active fractions from spent medium from *Shewanella* strain Hac334, using the same HPLC procedure. An analysis of both purified redox mediators and flavin standards by UV-visible spectroscopy resulted in identical spectra, confirming the redox mediators to be flavins. The identities of the purified redox mediators were also confirmed by LC-MS, giving identical molecular weights of 515 for the 28-minute fraction corresponding to FMN and of 377 for the 33-minute fraction corresponding to riboflavin. Standards for another common flavin, FAD, were also clearly resolved using the same HPLC protocol, with a retention time of 25 min, although it was not a major constituent of these extracellular samples.

In addition to identification by physical analyses, biological experiments also supported the hypothesis that native FMN and riboflavin were the purified extracellular redox mediators from cultures of *S. oneidensis* MR-1 and *Shewanella* strain Hac334. Equimolar (approximately 50 μM) solutions of purified FMN and riboflavin standards were tested in the dye reduction assay and resulted in similar rates of enhanced dye reduction noted with the purified compounds (all rates were in the range of 8.5 to 9.6 ± 1.3 μM dye/g protein · min). The culture supernatants of several other *Shewanella* strains (MR-1, J18 143, Os195, Hac318, Hac319, Hac326, Hac353, and Hac411 [see Materials and Methods for strain details]) were also analyzed by HPLC. All of them contained similar concentrations of FMN and riboflavin. Thus, the secretion of FMN and riboflavin would seem to be conserved among numerous *Shewanella* species.

Quantification of intracellular and secreted extracellular flavins. The production of the three flavins, i.e., FAD, FMN, and riboflavin, by cultures of *S. oneidensis* MR-1 grown for 24 h and their intra- and extracellular concentrations were assessed under aerobic and anaerobic growth conditions in the same defined minimal medium containing lactate and fumarate. Figure 1A shows the amounts of intracellular flavins detected, with FAD being the predominant flavin within cells, followed by FMN. Intracellular riboflavin was detected only in trace amounts. Figure 1B shows the amounts of extracellular flavins;

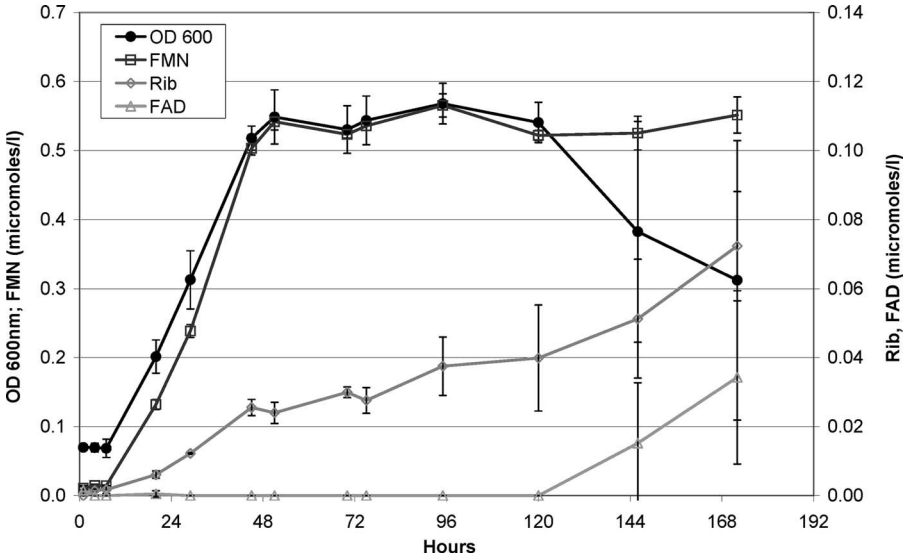

FIG. 2. Production of extracellular FMN (squares), riboflavin (Rib) (diamonds), and FAD (triangles) during anaerobic growth (circles) of *Shewanella oneidensis* MR-1 in a defined minimal medium containing 50 mM lactate and 100 mM fumarate. Data are averages for triplicate cultures.

no FAD was detected, while FMN was the predominant extracellular flavin, followed by riboflavin. The relative amounts of flavins per unit of biomass inside the cells and in the culture supernatants were similar under both anaerobic and aerobic growth conditions, although the secretion of flavins under aerobic conditions has no obvious advantages apart from potentially enhancing the exploitation of insoluble electron acceptors once the surrounding environment is depleted of oxygen. However, under both anaerobic and aerobic growth conditions, the amount of extracellular flavins was five times higher than that of intracellular flavins. This difference, as well as the fact that FAD was detected only inside the cells, suggests that FMN and riboflavin were actively secreted rather than released from porous or lysed dead cells. *Escherichia coli* strain JM109 cells, for comparison, had lower flavin concentrations both inside the cells and in the supernatant. Aerobically grown *S. oneidensis* MR-1 cultures contained 0.57 and 2.9 μmol of intracellular and extracellular flavins/g protein, respectively, while the *E. coli* culture grown under the same conditions contained only 0.33 and 0.7 μmol flavins/g protein, with riboflavin being the predominant extracellular flavin. The hypothesis that FMN and riboflavin are actively secreted by *Shewanella* cells is also supported by a time course analysis of extracellular flavins produced during anaerobic growth (Fig. 2). FMN secretion was strongly correlated with cell proliferation and ceased when growth stopped, after approximately 50 h. Riboflavin secretion was less strongly correlated with growth and happened throughout the cultivation period. FAD, in contrast, was detected only after cell lysis was noted, at 120 h of cultivation. Flavins were also produced under Fe(III)-reducing conditions. In cultures of *S. oneidensis* MR-1 and *Shewanella* sp. strain Hac334 with Fe(III)-citrate as the electron acceptor, the concentrations of extracellular flavins were highest after 168 h and reached a total flavin concentration of 0.03 μM. With poorly soluble Fe(III) oxide as the electron acceptor, the concentrations were highest after 246 h and reached a total flavin concentration of 0.01 μM. In the case of Fe(III)-citrate cultures, the specific flavin production was 2.0 and 1.8 μM/g protein for the MR-1 and Hac334 strains, respectively, and thus about in the same range as in the MR-1 cultures grown with fumarate as the electron acceptor (2.9 μM/g protein) (see above). With the Fe(III) oxide cultures, the determination of the exact amount of biomass was impaired by low protein concentrations and a high background of Fe(II), but as an estimate, the specific flavin concentrations were approximately as high as those of the Fe(III)-citrate cultures.

Flavin secretion by *Shewanella* strains. In order to determine if other *Shewanella* species share the ability to secrete flavins in actively growing cultures, 11 phylogenetically distinct *Shewanella* strains were studied under both aerobic and anaerobic conditions. To determine if other gammaproteobacteria also secrete flavins and utilize them as electron shuttles, three *Pseudomonas* and one *Escherichia* species were also studied. As shown in Fig. 3, all 11 *Shewanella* strains secreted FMN and riboflavin, with 0.5 to 0.9 μM extracellular FMN/g cell protein measured after 49 h of aerobic growth. The concentrations of riboflavin ranged from 0.1 to 0.3 μM/g protein. The *Pseudomonas* and *Escherichia* species also secreted flavins, with all but the *P. putida* strain secreting not only FMN and riboflavin but FAD as well.

Under anaerobic growth conditions, the *Shewanella* strains secreted higher specific concentrations of FMN, i.e., between 0.6 μM/g protein (Os155) and 1.8 μM/g protein (Hac326). The specific riboflavin concentrations were much lower than those obtained under aerobic growth conditions, with the highest concentration of 0.08 μM/g protein found in MR-1 cultures.

All 11 *Shewanella* strains were also found to be capable of reducing the azo dye Direct Blue 53 under anaerobic conditions, and decoloration rates were accelerated by the addition of riboflavin. The *Pseudomonas* and *Escherichia* strains were not able to reduce the azo dye, with or without riboflavin

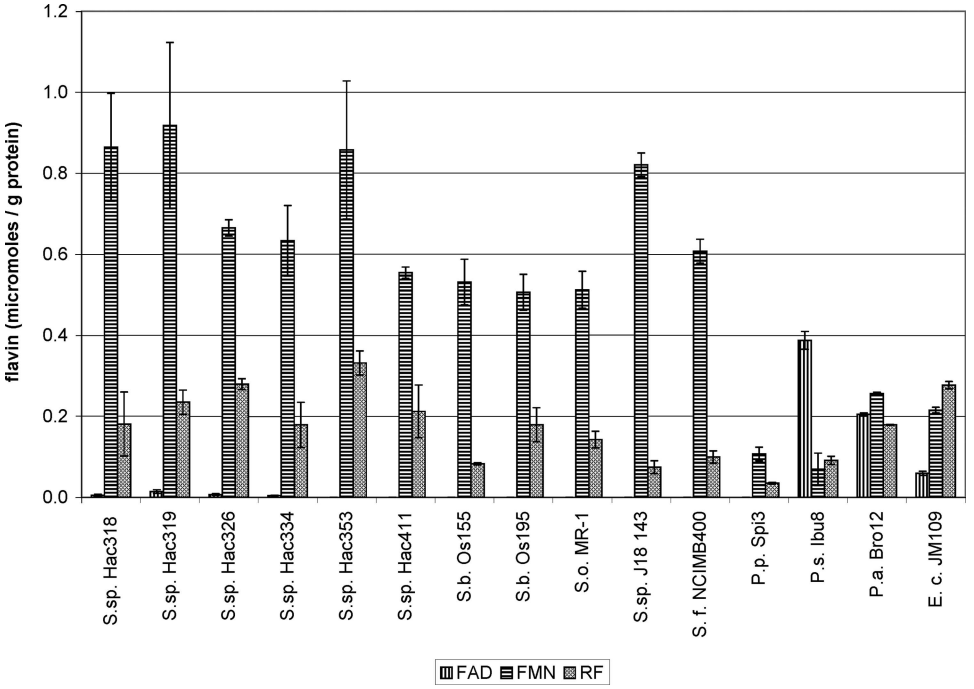

FIG. 3. Extracellular flavin yields in cultures of *Shewanella* and *Pseudomonas* species after 49 h of aerobic growth in minimal medium containing 50 mM lactate and 100 mM fumarate. Data are averages for triplicate cultures. S.sp., *Shewanella* sp.; S.b., *S. baltica*; S.o., *S. oneidensis*; S.f., *S. frigidimarina*; P.p., *P. putida*; P.s., *P. stutzeri*; P.a., *P. aeruginosa*; E.c., *E. coli*; RF, riboflavin.

present (data not shown), suggesting that they were unable to use secreted flavins as redox mediators for dye reduction.

Secreted flavins promote reduction of poorly soluble Fe(III) oxides. To demonstrate that FMN and riboflavin can transfer electrons to poorly soluble Fe(III) oxide, 1 mM of FMN or riboflavin was chemically reduced with hydrogen using palladium as the catalyst and then incubated with 6 mM of poorly soluble Fe(III) oxide. This resulted in the formation of 2 mM of Fe(II). Control experiments with ddH_2O resulted in no Fe(III) reduction. This demonstrates that one flavin molecule can transfer two electrons to reduce two molecules of Fe(III) oxide.

To investigate whether the full range of flavins produced intracellularly and extracellularly by *S. oneidensis* MR-1 had the capacity to act as redox mediators during microbial Fe(III) reduction, *S. oneidensis* MR-1 cells were grown anaerobically until mid-log phase, with either fumarate or Fe(III)-citrate as the electron acceptor, washed, and resuspended in a bicarbonate buffer of pH 7.0 containing 100 mM lactate as an electron donor, 20 mM of either poorly crystalline insoluble Fe(III) oxide or soluble Fe(III)-citrate, and 10 μM of the potential electron shuttle FAD, FMN, or riboflavin or the known electron shuttle anthraquinone-2,6-disulfonate (AQDS). We hypothesized that the flavin molecules and AQDS would enhance the reduction of the extracellular electron acceptor, the poorly crystalline Fe(III) oxide, obviating the need for direct contact between the cell and the mineral surface. This was indeed the case (Fig. 4). Here the reduction rate of the Fe(III) oxide was increased by a factor of 18, to approximately 0.2 mmol min^{-1} g $protein^{-1}$, in the presence of 10 μM of the electron shuttles. All three flavins were equally effective as electron shuttles and gave similar results to those with AQDS, the "benchmark" exogenous electron shuttle used in many other studies to accelerate microbial Fe(III) and azo dye reduction (11, 22). In addition, the rates of reduction of soluble Fe(III)-citrate were identical for both fumarate- and Fe(III)-citrate-grown cells, regardless of the addition of an electron shuttle, showing that although biological flavins and AQDS enhance the rate of reduction of insoluble Fe(III) oxides, they are not required for maximal rates of reduction of soluble, highly bioavailable Fe(III)-citrate that can diffuse into the cell and potentially cross the outer membrane to penetrate the periplasm. Indeed, the maximum specific rate of reduction of Fe(III)-citrate was 609 μmol Fe(III) $minute^{-1}$ g $protein^{-1}$, while the rate of reduction of poorly crystalline Fe(III) oxide was $<2\%$ of this value in the absence of added electron shuttles but rose to 39% of this value with 10 μM of added flavins. These results indicate that *Shewanella* cells are able to utilize spatially removed extracellular electron acceptors, including Fe(III) and potentially Mn(IV) oxides, via the secretion of flavin molecules.

To confirm that flavins enhanced Fe(III) reduction even in the submicromolar concentration range likely to be encountered in natural environments, high-density cultures of washed cells of *Shewanella* strain Hac334 (optical density at 600 nm, 2.9) were resuspended with poorly crystalline Fe(III) oxide, and the initial rate of Fe(III) reduction was measured in the presence of different concentrations of riboflavin. This approach also made it possible to quantify Fe(III) reduction rates within 1 hour of inoculation, under conditions relatively unaffected by cellular growth or flavin secretion. The addition of riboflavin had a discernible promoting effect on the reduction

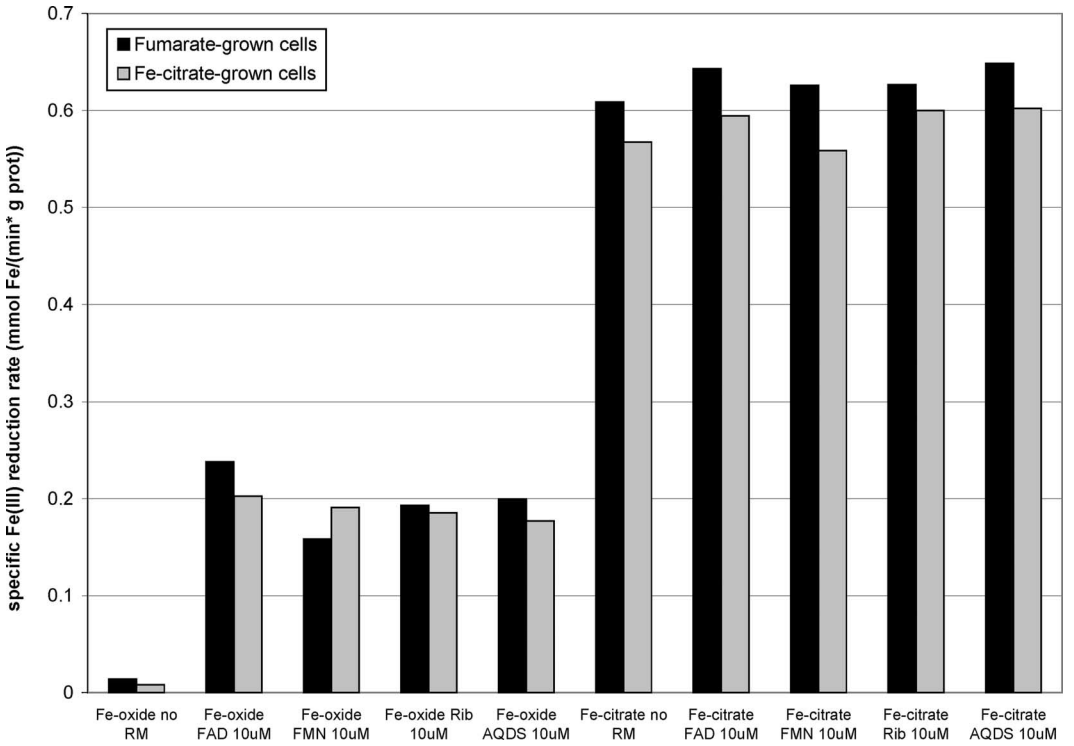

FIG. 4. Fe(III) reduction rates of *Shewanella oneidensis* MR-1 cells grown with fumarate (black) or Fe(III)-citrate (gray), with poorly soluble Fe(III) oxide and Fe(III)-citrate as substrates and in the absence of redox mediators (RM) or with 10 μM of the redox mediator FAD, FMN, riboflavin (Rib), or AQDS. Data are averages for triplicate cultures.

of poorly crystalline Fe(III) oxide at concentrations of 0.1 μM and higher (Fig. 5). In the presence of 1 μM riboflavin, the maximum specific rate of Fe(III) reduction over the first 30 min of the experiment was twofold higher than that for riboflavin-free controls (62 μmol compared to 30 μmol Fe(III) $minute^{-1}$ g $protein^{-1}$), and it was eightfold higher (241 μmol Fe(III) $minute^{-1}$ g $protein^{-1}$) in the presence of 100 μM riboflavin. As suggested by the results shown in Fig. 4, the rates of reduction of soluble Fe(III)-citrate were identical for all cultures, regardless of the concentration of the electron shuttle added.

In addition to stimulating the specific rate of reduction of Fe(III) oxides by pregrown cultures, extracellular flavins were also shown to enhance the growth of *Shewanella* species on insoluble Fe(III) minerals (data not shown). For example, when low densities of *Shewanella* strain Hac334 cells (4.0×10^2 CFU/ml) were inoculated into nitrogen-flushed, oxygen-free medium containing 100 mM of lactate as the electron donor and 35 mmol $liter^{-1}$ of poorly crystalline Fe(III) oxide as the electron acceptor, the growth rate and cell yield were positively correlated with the amount of extracellular flavin over a concentration range of 0.01 μM to 100 μM. For example, with the addition of 10 μM riboflavin as a model flavin compound, there were 1.5×10^7 CFU/ml at the end of the growth phase (49 h), compared to 7.8×10^6 CFU/ml with the addition of 1 μM riboflavin and 3.7×10^6 CFU/ml in the absence of the added flavin. Thus, extracellular flavins were shown to promote growth coupled to the dissimilatory reduction of poorly crystalline insoluble Fe(III) oxide through functioning as an electron shuttle.

DISCUSSION

Although the secretion of endogenous electron shuttles to promote the dissimilatory reduction of poorly soluble Fe(III) oxides has been proposed for at least two phylogenetically distinct Fe(III)-reducing bacterial species, namely, *Shewanella* (19) and *Geothrix* (18), to date the identity of the electron shuttle has remained elusive for both organisms. For *Shewanella* species, which are some of the most intensively studied organisms able to conserve energy for growth through the dissimilatory reduction of extracellular mineral phases, several possible candidates have been discussed. For example, melanin has been proposed as an endogenous electron shuttle for *Shewanella algae*, enhancing the reduction of Fe(III) minerals (28). However, production of melanin occurs only under aerobic conditions with tyrosine as a precursor, and it is therefore questionable whether this electron shuttle plays a role in the reduction of Fe(III) oxides in anoxic zones in the environment. *S. oneidensis* MR-1 was reported to release an alternative redox-active low-molecular-weight compound, possibly a quinone, that reversed the phenotype of mutant strains that were deficient in menaquinone synthesis and unable to reduce the artificial redox mediator AQDS (19). Although it was noted that this excreted compound could function by enabling the cells to synthesize menaquinone, it was also hypothesized that

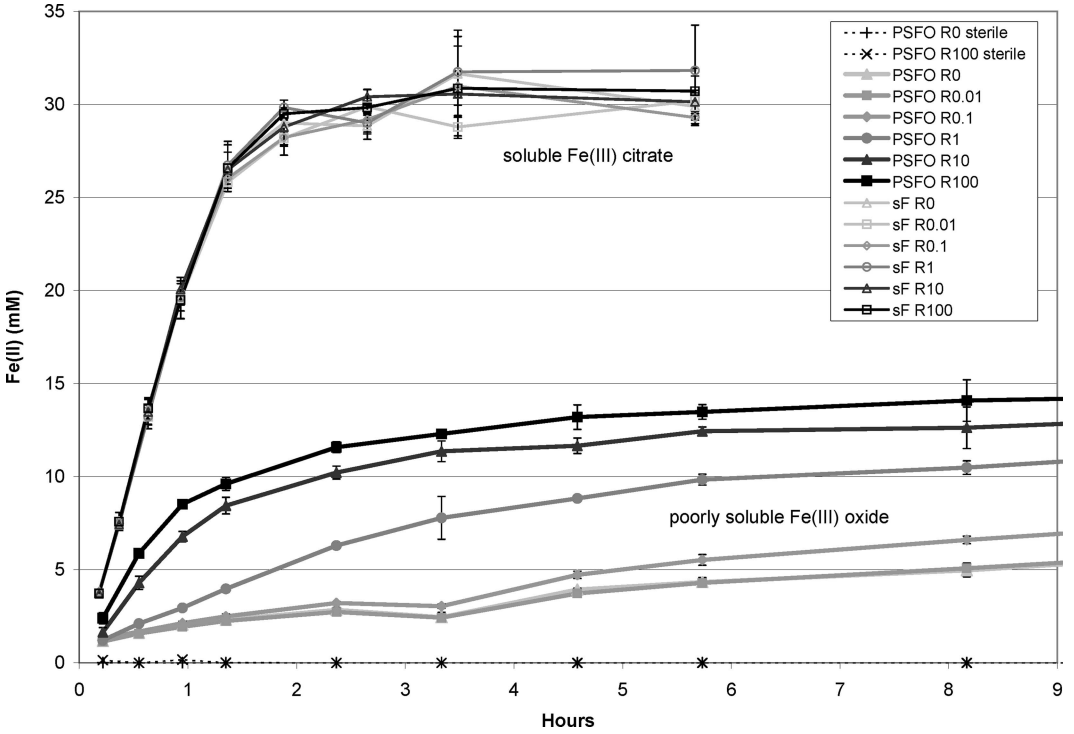

FIG. 5. Reduction of poorly soluble Fe(III) oxide (PSFO; closed symbols) and soluble Fe(III)-citrate (sF; open symbols) by cells of *Shewanella* strain Hac334. The concentration of riboflavin added to the assay mixtures ranged from 0 to 100 μM (R0 to R100). Data are averages for triplicate cultures.

it could be involved directly in extracellular electron transfer. This hypothesis was challenged subsequently (15), when it was noted that the addition of culture supernatants of *S. oneidensis* MR-1 restored menaquinone synthesis in a menaquinone synthesis-negative mutant. Furthermore, it was considered unlikely that menaquinone or a menaquinone-like compound is the secreted electron shuttle produced by *Shewanella* species, as proteins downstream of the cellular menaquinone pool (e.g., periplasmic CymA) are required for the reduction of many electron acceptors, including Fe(III) oxides (15).

The compounds identified in this study, FMN and riboflavin, are distinct from those discussed in previous studies on the basis of solubility, UV-visible spectra, and production under a wider range of growth regimens and are excellent candidates for secreted soluble electron mediators to promote the reduction of extracellular electron acceptors. Riboflavin occurs almost exclusively as a constituent of the two flavin prosthetic groups of flavoproteins, i.e., FAD and FMN. These three flavins have the ability to undergo oxidation-reduction reactions through the stepwise reversible addition of two electrons via the semiquinone form to the colorless reduced form (3). With redox potentials (E_0') of −219 mV (FMN and FAD) and −208 mV (riboflavin) (reviewed by van der Zee [29]), flavins are more electronegative than the redox couple of poorly crystalline Fe(III) oxide (ferrihydrite)/Fe^{2+} (−100 to +100 mV) (reviewed by Straub et al. [27]). Thus, flavins have the potential to act as efficient extracellular redox mediators for the reduction of poorly soluble Fe(III) oxide at neutral pH. This, in turn, could give *Shewanella* species and other microorganisms that not only secrete flavins but also utilize them as electron shuttles an advantage in environments that contain poorly soluble Fe(III) compounds but lack exogenous redox mediators such as humics (27). This is important, as this and numerous other studies have shown that without electron shuttles, *Shewanella* cells reduce poorly soluble Fe(III) oxide very slowly.

Given the almost exclusive occurrence of FAD inside the cells and the concentrations of extracellular FMN and riboflavin being up to 30 times higher than those of intracellular FMN and riboflavin, it is unlikely that FMN and riboflavin are released through cell lysis; instead, it is likely that they are actively secreted. Indeed, although microbial cells are relatively impermeable to external flavins, efficient secretion of internal flavins, especially riboflavin, has been noted previously (4) but never before associated with enhancing respiration by using insoluble extracellular electron acceptors. It is curious that the predominant secreted flavins are FMN and riboflavin, although the major intracellular flavin is FAD. Little is known about the molecular mechanisms involved in flavin transport across cell membranes, but a report on the riboflavin import protein RibU in *Lactococcus lactis*, which binds riboflavin and FMN, but not FAD (6), can be taken as an example of differential flavin transport across membranes.

Despite their potential role as extracellular electron shuttles for *Shewanella* cells, it is worth noting that the amounts of intracellular flavins found in *S. oneidensis* MR-1 cells were in the range of those recorded for other model organisms, i.e., 0.06 to 0.72 μmol/g protein, predominantly FAD and FMN (4), or 0.33 μmol/g protein (predominantly FAD and FMN [this study]) in *E. coli*, compared to 0.41 to 0.57 μmol/g protein in *S. oneidensis* MR-1 cells. Also, the concentration of flavins se-

creted by anaerobically grown *Shewanella* cells is relatively low in comparison to the amounts of riboflavin secreted by overproducing strains of *Clostridium acetobutylicum* and *Eremothecium ashbyii* (0.6 μM and 260 to 6,600 μM, respectively) (reviewed in reference 4), although comparison is complicated by the different cultivation conditions used for these organisms. However, the concentrations of flavins secreted by planktonic cultures of *Shewanella* cells reported here (0.1 to 0.6 μM) were shown to be high enough to have a significant effect on electron transfer to poorly soluble Fe(III) oxides. Furthermore, the local concentrations of secreted flavins in microenvironments such as biofilms or micropores in minerals are potentially much higher. Thus, although we have not quantified flavin production by *Shewanella* cells immobilized in biofilms, the secretion of flavins under conditions of oxygen limitation would potentially enhance the considerable metabolic diversity of this organism, which can colonize redox boundaries in dynamic systems through the utilization of a range of electron acceptors, including insoluble Fe(III) oxides. The phenomenon that *Shewanella* cells secrete flavins equally efficiently under both aerobic and anaerobic conditions could be interpreted as energetically wasteful, since electron shuttles are not needed when oxygen serves as the terminal electron acceptor. Surprisingly, the ability to secrete flavins during aerobic growth seems to be quite a widespread phenomenon among gammaproteobacteria, with the secretion rates of *E. coli* and *Pseudomonas* species, not known to utilize flavins as redox mediators under anaerobic conditions, found to be within the same order of magnitude as those of the *Shewanella* strains that we tested. It is not clear why the *E. coli* and *Pseudomonas* strains would secrete these flavin molecules under aerobic conditions, but *Shewanella* cells are known to live in interfacial environments, such as the Baltic Sea's oxic-anoxic interface (2), where conditions can change quickly from oxic to anoxic, and the flavins secreted during aerobic growth could play a useful role in anaerobic respiration using insoluble Fe(III) or Mn(IV) oxides. At the microbe-mineral interface, the metabolic expense of secreting FMN or riboflavin may be an energetically favorable process, as it could be used in multiple rounds of extracellular electron shuttling in this localized environment.

Several intriguing aspects of this study warrant further investigation. First, the ability to secrete flavins is not exclusive to *Shewanella* (4, 9), and other organisms may well have the potential to harness their redox-mediating capabilities. If this is so, then there is a possibility that these secreted compounds could play a far more extensive role in a range of redox processes in the environment. Finally, in extracellular electron transfer mediated by secreted FMN and riboflavin, we have identified a potentially important mechanism that could be enhanced through genetic manipulation to facilitate the wide range of innovative biotechnological processes that utilize *Shewanella* cells, including the bioremediation of organics (22), metals, and radionuclides (10) and energy production in microbial fuel cells (20). For example, we have shown recently that riboflavin, FMN, and FAD can act as extracellular electron shuttles in microbial fuel cells, resulting in up to fivefold higher current and power densities (S. Velasquez Orta, I. M. Head, T. P. Curtis, K. Scott, J. R. Lloyd, and H. von Canstein, unpublished data).

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